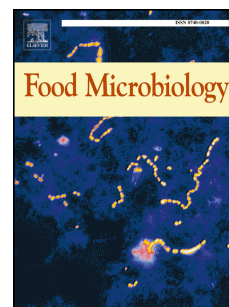


Accepted Manuscript

Redefining the effect of salt on thermophilic starter cell viability, culturability and metabolic activity in cheese

C.D. Hickey, V. Fallico, M.G. Wilkinson, J.J. Sheehan



PII: S0740-0020(17)30330-1

DOI: [10.1016/j.fm.2017.08.015](https://doi.org/10.1016/j.fm.2017.08.015)

Reference: YFMIC 2851

To appear in: *Food Microbiology*

Received Date: 6 April 2017

Revised Date: 8 August 2017

Accepted Date: 22 August 2017

Please cite this article as: Hickey, C.D., Fallico, V., Wilkinson, M.G., Sheehan, J.J., Redefining the effect of salt on thermophilic starter cell viability, culturability and metabolic activity in cheese, *Food Microbiology* (2017), doi: 10.1016/j.fm.2017.08.015.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Redefining the effect of salt on thermophilic starter cell viability,
culturability and metabolic activity in cheese**

C. D. Hickey,^{a,b,*} V. Fallico,^{a,*} M. G. Wilkinson,^b and J.J. Sheehan^a

^a*Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland*

(Tel.: +353(0) 25 42232; e-mail: Cian.Hickey@teagasc.ie; Vincenzo.Fallico@teagasc.ie;
Diarmuid.Sheehan@teagasc.ie)

^b*University of Limerick, Castletroy, Limerick, Ireland*

(Tel.: +353 (0) 61 213440; e-mail: Martin.Wilkinson@ul.ie)

**Corresponding authors*

Abstract

This study investigated the differential effect of salt concentration in the outside and inside layers of brine salted cheeses on viability, culturability and enzyme activity of starter bacteria. The high-salt environment of the outside layer caused a sharp decrease in *L. helveticus* viability as measured by traditional plate counts. Remarkably, this was associated with lower release of intracellular enzymes (LDH), reduced levels of proteolysis and larger membrane integrity as measured by flow cytometry (FC) following classical Live/Dead staining. FC analysis of light scattering properties highlighted a significant reduction in size and granularity of the microbiota located in the cheese surface, suggestive of cell shrinkage and condensation of internal macromolecules probably due to hyperosmotic stress. The microbiota of the cheese surface were found to experience greater oxidative stress, as measured by FC analysis of the total levels of reactive oxygen species, compared to that of

the interior layer. These results lead us to postulate that the physiology and health status of the microbiota were significantly different in the outer and inner layers of the cheese. The hyperosmotic environment of the outer layer resulted in reduced cell lysis, as measurable by assays based upon membrane integrity, but rather triggered cell death via mechanisms involving cell shrinkage and ROS-mediated damage of vital intracellular components. This study challenges the current thinking on how salt controls microbial activity in ripening cheese, especially in cheeses which are brine salted as local variations in biochemical ripening indices can differ significantly from the outside to the inside of a ripening cheese.

Keywords

Salt concentration; Osmotic pressure; Starter viability; Flow cytometry; Membrane integrity

1. Introduction

Salt has been used as a food preservative since prehistoric times and has worked especially well, combined with fermentation and dehydration, as is the case with cheese production (Guinee & Fox, 2004). Salt content directly influences cheese flavour, provides sodium, essential for control of blood pressure and healthy cell function within the body, and crucially acts as a preservative. Salt lowers the water activity within the cheese matrix and subsequently controls microbial growth, enzyme activity, extent of protein hydration and aggregation along with rheological and cooking properties of cheese (Guinee, 2004; Guinea & Fox, 2004). Brine salted cheeses differ from their dry salted counterparts in that salt uptake is gradual and salt equilibrium occurs over a period of days/weeks/months (Sutherland,

2002), unlike dry salted cheeses where salt is relatively evenly distributed during the salting process (Fox, Guinee, Cogan, & McSweeney, 2000).

The diffusion coefficient (D^*) of salt in brine salted cheeses has been investigated previously (Floury, Jeanson, Aly, & Lortal, 2010; Floury, Madec, Waharte, Jeanson, & Lortal, 2012; Gomes, Vieira, & Malcata, 1998; Guinee, 2004; Lee, Olson, & Lund, 1980; Pajonk, Saurel, & Andrieu, 2003; Payne & Morison, 1999; Turhan & Kaletunc, 1992) with the D^* generally estimated at $\sim 0.2 \text{ cm}^2/\text{day}$, but ranges from $0.1\text{-}0.45 \text{ cm}^2/\text{day}$, depending on curd temperature and moisture composition and brining conditions. Brining of cheese results in a net movement of Na^+ and Cl^- from the brine into the outer layer of the cheese block due to osmotic pressure (Guinee, 2004; Turhan & Gunasekaran, 1999). This osmotic pressure and high salt concentration of the brine (15-23% NaCl) which initially locates at the outer layer of the cheese is of interest in relation to its effect on cell viability, NSLAB growth, enzyme activity and possible development of ripening hotspots due to localised variations in salt in moisture levels (Fox, et al., 2000).

Salt has long been associated with the control of lactose metabolism, curd pH and starter bacteria growth and rate of lysis (Wilkinson, Guinee, & Fox, 1994a; Wilkinson, Guinee, O'Callaghan, & Fox, 1994b; Yanachkina, McCarthy, Guinee, & Wilkinson, 2016). The subsequent autolysis of bacterial cells and release of intracellular enzymes is heavily involved in the development of cheese matrix structure and flavour (Doolan & Wilkinson, 2009; Rulikowska, et al., 2013; Sheehan, Cuinn, FitzGerald, & Wilkinson, 2006; Thomas & Pearce, 1981; Wilkinson, et al., 1994a; Wilkinson, et al., 1994b). The effect salt concentration, resulting from brine salting of cheese, has on these crucial intracellular enzymes and their functions has yet to be fully investigated.

Flow cytometry (FC) enables the assessment of various structural and functional cell properties, often leading to a deeper characterization of the physiological heterogeneity of a microbial population (Díaz, Herrero, García, & Quirós, 2010). In combination with fluorescent probes sensitive to the integrity of the cell membrane, FC has been used for the identification of cell viability and predicting levels of cell lysis during cheese ripening (Bunthof, van Schalkwijk, Meijer, Abee, & Hugenholtz, 2001; Comas-Riu & Rius, 2009; Doolan & Wilkinson, 2009; Doolan, Nongonierma, Kilcawley, & Wilkinson, 2014; Sheehan, O'Loughlin, O'Cuinn, FitzGerald, & Wilkinson, 2005; Sheehan, O'Cuinn, FitzGerald, & Wilkinson, 2009). The gap between FC results and viable cell counts via traditional plating on various selective agars has long been a major issue with suggested overestimations of cell count levels in comparison to counts using FC (Bunthof & Abee, 2002).

In cheese manufacture, dry or brine salting is used to control the proliferation of spoilage and undesired microbiota but will obviously impact on the physiology of starter bacteria as well. Transcriptomic studies have shown the induction of various oxidative stress response genes and enzymic antioxidants in bacteria exposed to salt stress (den Besten, Mols, Moezelaar, Zwietering, & Abee, 2009; Tsuzuki, et al., 2011), thereby pointing to the potential generation of reactive oxygen species (ROS). When ROS accumulate to levels exceeding the scavenging capacity of the bacterial cell, a state of oxidative stress is generated where excess ROS break nucleic acids, carbonylate proteins, peroxidate lipids, inhibit enzymes and ultimately lead to cell death. Consequently, FC is utilised in this study to compare total cell numbers with traditional plate count methods, along with assessing bacterial cell membrane integrity, morphology and reactive oxygen species (ROS) levels in order to determine how bacteria react to environmental stressors (i.e. salt).

To the authors knowledge, no study to date has investigated the effect of salt gradients in brine salted cheeses on bacterial viability and subsequent enzymatic activity within the

cheese matrix. The objectives of this study were to (i) establish differing salt concentrations and cheese compositions at localised level (ii) establish the influence of varying salt concentrations on microbial activity (iii) establish the influence of varying salt concentrations on microbial metabolic activity and (iv) evaluate the use of FC and fluorescent probes in comparison to plate counts in the study of cheese microbiology.

2. Materials and Methods

2.1 Starter strains

Thermophilic starter cultures typically used in Swiss-type cheese manufacture (Scott, 1981), i.e., *L. helveticus* LHB02 (LH) and *S. thermophilus* TH3 (ST) were sourced (Chr. Hansen Ltd, Little Island, Co. Cork, Ireland) as individual frozen DVS concentrates and stored at -80 °C until cheese manufacture.

2.2 Cheese manufacture and sampling procedure

Three replicate cheesemaking trials, each consisting of four vats of 380 kg cheese milk were undertaken over a 6 month period. Raw milks were obtained from a local dairy company, standardised to a protein to fat ratio of 1.01:1, held overnight at < 10 °C, pasteurized at 72 °C for 15 s, and pumped at 36 °C into cylindrical, jacketed, stainless steel vats (500L) with automated variable speed cutting and stirring equipment (APV) Schweiz AG, Worb, Switzerland).

Calcium Chloride (CaCl₂) was added at 0.022 % (v/v). The milks were inoculated with 1 of 3 starter blends.

LH: 0.015% w/v *Lactobacillus helveticus* (LHB02)

ST: 0.015% w/v *Streptococcus thermophilus* (TH3)

ST + LH. 0.01% w/v *Streptococcus thermophilus* (TH3) & 0.005% w/v *Lactobacillus helveticus* (LHB02)

After a 60 min ripening period, an *Cryphonectria parasitica* rennet (Thermolase (625 IMCU/ ml), Chr. Hansen Ltd., Little Island, Co. Cork, Ireland), diluted ~1:10 with de-ionised water, was added at a level of 3.7 mL 100 kg⁻¹ milk. A coagulation period of ~30 min was allowed prior to the mechanical cutting of the coagulum. After a 5 min healing period, the curd/whey mixture was stirred and cooked by steam injection into the jacket of the vat. Curds were cooked at a rate of 0.5 °C min⁻¹ from 36 to 45 °C and at 1 °C min⁻¹ from 45 °C to maximum scald (50 °C).

Curds were pitched at pH 6.3, pre-pressed under whey at 5.4 kPa for 5min after which the curd was placed in 10 kg round moulds and pressed under increasing pressure up to 6 kPa. At pH 5.3, the cheeses were placed for 60 hrs in either a saturated brine solution (22 % w/w NaCl, 0.2 % w/w Ca, pH 5.2) or in a brine solution containing 18 % w/w NaCl, 0.2 % w/w Ca at pH 5.2 solution and at 18-20 °C (controlled via calcium chloride addition). On removal from the brine, all cheeses were vacuum packed and ripened at 8 °C for 26 d (outlined in supplementary Table. 1). Cheeses were sampled by removing a wedge aseptically from each wheel. Each wedge was separated into 2 samples based on location. The outer 2.5 cm was removed from the top, bottom and edge of the cheese wedge. The outer most 1.5 cm was grated and used as the outside sample point, while a similar quantity of the inner portion (w/w) of the cheese was grated and represented the inside portion.

2.3 Enumeration of starter and non-starter bacteria

During cheese manufacture, curd samples were removed aseptically pre and post brining. Further samples were acquired aseptically at 4, 7, 11, 18 and 26 d of ripening. Samples were

placed in a sterile stomacher bag, diluted 1:10 with sterile 2 % (w/v) trisodium citrate and homogenized in a stomacher (Stomacher, Lab-Blender 400, Seward, Thetford, Norfolk, UK) for 10 min. Further serial dilutions were prepared as required using maximum recovery diluent (MRD).

Viable *S. thermophilus* cells were enumerated on lactose-M17 agar after aerobic incubation at 42 °C for 3 d (Terzaghi & Sandine, 1975), *L. helveticus* cells were enumerated on MRS agar pH 5.4 after anaerobic incubation for 3 d at 42 °C (IDF, 1988a), Enterococci cells were enumerated on kanamycin aesculin azide (KAA) agar aerobically at 37 °C for 24hrs and non-starter lactic acid bacteria (NSLAB) were enumerated on LBS agar with an LBS agar overlay at 30 °C for 5 d (Rogosa, Mitchell, & Wiseman, 1951).

2.4 Cheese composition

Grated cheese samples were analysed at 0, 4, 7, 11, 18 and 26 d of ripening for total salt (IDF, 1988b), protein (IDF, 1993), moisture and fat via nuclear magnetic resonance (NMR) (Fast Trac analysis system, CEM Microwave technology Ltd., Dublin, Ireland). Cheese pH was measured by preparing a cheese slurry from 20 g of grated cheese combined with 12 g of H₂O (45-55 °C).

2.5 Enzymatic activity analysis

Autolysis of starter cultures during cheese ripening was monitored by assaying in triplicate for the release of the intracellular enzymes lactate dehydrogenase (LDH) in cheese extracts using a modification of the methods by Cogan, O'Dowd, and Mellerick (1981) and Wittenberger and Angelo (1970), which measure the decrease in absorbance at 340 nm resulting from the pyruvate-dependant oxidation of reduced nicotinamide adenine dinucleotide (NADH). The reaction mixture contained 100 µL of 300 mm sodium pyruvate,

100 μ L of 4.5 mM NADH and 100 μ L of sample, with final reaction volume made up to 3 mL using 0.2 M Tris-maleate buffer (pH 7.0). Reaction was initiated by the addition of sodium pyruvate. Sampling time points correspond to those for starter enumeration. LDH activity was expressed as LDH units per mL of extract, where one unit was defined as the amount of enzyme required to catalyse the oxidation of 1 mM NADH per min.

2.6 FC analysis of cheese microbiota

A novel, enzyme-free protocol was utilised in this study, to prepare a cheese bacterial extracts of suitable quality for FC analysis. Cheese (10 g) was weighed into the sample compartment of a sterile filter bag (280 μ m pore size) (Interscience, France), diluted ten-fold with 2 % trisodium citrate (VWR, Ireland) and homogenized at high speed for 10 min using a stomacher (Stomacher, Lab-Blender 400, Seward, Thetford, Norfolk, UK). The cheese homogenate was transferred from the filtrate compartment of the 280 μ m filter bag to the sample compartment of a sterile filter bag (63 μ m pore size) (Interscience, France) and stomached at high speed for further 2 min. The microbiota were recovered from the cheese homogenate by centrifugation at 2,790 g for 10 min at 4 °C. Residual fat was removed using sterile cotton buds, the bacterial pellet was re-suspended in 1 ml of 0.2 μ m-filtered Maximum Recovery Diluent (MRD) (Oxoid, Ireland) containing 0.02 % Tween 20 (Invitrogen, Ireland) and 1 mM EDTA (Sigma, Ireland). The same buffer was used to dilute the bacterial sample to an appropriate cell concentration to allow for the analysis of less than 1,000 cellular events per min and avoid coincidence at the flow cytometer.

Analysis of membrane integrity was carried out by staining the bacterial samples with 6.68 μ M Syto 9 and 40 μ M Propidium Iodide (PI) from the Live/Dead[®] BacLight[™] viability kit (Molecular Probes, USA) for 15 min at 37 °C, under agitation (400 rpm) in the dark. Consequently, live cells with an intact membrane generally exhibit green fluorescence as they

contain only Syto 9 whereas dead cells with a compromised membrane are stained red by PI. Injured cells with a permeabilized membrane will contain both dyes in a ratio proportional to the degree of membrane damage and will exhibit orange fluorescence (Cronin, 2015).

Data acquisition and analysis were carried out using the BD Accuri C6 software v. 1.0.2 (Becton Dickinson, Belgium). The overall cheese microbiota was identified based on light scattering properties on forward scatter (FSC) vs side-angle scatter (SSC) biplots and a gating strategy was used to discriminate from background particles and cell debris (supplementary Fig. 1). FSC and SSC signals represent a measure of cell size and internal complexity, respectively (Díaz, et al., 2010). The green fluorescence of Syto 9 positive cells were monitored on the FL1 channel (530/30 nm) whereas red-fluorescent PI positive cells were detected on the FL3 channel (> 670 nm long pass). The counts or events measured for each gated population were converted to colony forming units (CFU) per gram of cheese sample analyzed by using the following equation:

$$\text{CFU/ g cheese} = \frac{(\text{gate counts}) \times (\text{sample dilution factor}) \times 1000}{(\text{microlitres of sample analyzed})}$$

2.7 Proteolysis

2.7.1 Primary and secondary proteolysis

Levels of primary proteolysis, expressed as % pH 4.6 soluble N/TN (pH 4.6 SN/TN), was measured by the methods described by Kuchroo and Fox (1982) and Hickey, Kilcawley, Beresford, Sheehan, and Wilkinson (2007). Nitrogen was determined by the macro-kjeldahl method (IDF 1993). Secondary proteolysis was determined via the quantification of free amino acids present in a pH 4.6 SN extract at 0 and 26 days of ripening. Analysis was carried

out as described by Fenelon, O'Connor, and Guinee (2000). Samples were deproteinised by mixing equal volumes of pH 4.6 SN extract and trichloroacetic acid (240 g kg⁻¹). Free amino acids were separated using ion-exchange chromatography with post-column ninhydrin derivatisation and visible colorimetric detection. Values reported are the means of three replicate trials.

2.7.2 Urea-polyacrylamide gel electrophoresis (Urea-PAGE)

Urea-polyacrylamide gel electrophoresis was performed on cheese samples after 26 d of ripening, using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, Herts., UK) and the stacking gel system described by Andrews (1983). The gels were stained directly by the method of Blakesley and Boezi (1977) using Coomassie Brilliant Blue G250.

2.8 Statistical analysis

Analysis of variance (ANOVA) was performed using the general linear model (GLM) procedure (SAS, 1995) where the effects of treatments (sample location and starter type) and replicates were estimated for response variables relating to cheese composition. Duncan's multiple comparison tests was used as a guide for paired comparisons of the treatment means. The level of significance was determined at $P < 0.05$.

Split plot design was used to determine the effects of sample location, salt gradient, ripening time and their interaction on microbial (*S. thermophilus* and *L. helveticus* viability, NSLAB viability during ripening) and biochemical (LDH enzyme activity, pH, total and individual free amino acids) parameters measured at regular intervals during manufacture and/or ripening.

ANOVA for the split plot design was carried out using a GLM procedure (SAS, 1995). Statistically significant differences ($P < 0.05$) between means were determined by Fisher's least significant difference. Application of a split plot design to determine the effects of sample location, salt gradient, ripening time and their interaction on the various parameters were performed as reported by (Sheehan, Fenelon, Wilkinson, & McSweeney, 2007).

3. Results and Discussion

3.1.1 Chemical composition

Manufacture of the cheeses using starters in combination or using each starter individually did not significantly affect cheese protein, moisture, MNFS, fat, FDM, salt or S/M contents (Table 1). Brining of cheeses in a 22 % compared to 18 % NaCl concentration (IDF, 1988b) showed no significant effect on salt uptake by the cheeses. Cheese manufactured with *S. thermophilus* only had a significantly higher pH ($P < 0.05$) than in LH or ST/LH cheeses. This higher pH may be due to the significant accumulation ($P < 0.001$) of galactose, produced due to the activity of *S. thermophilus* during the early stages of ripening, as was observed in this study (results not shown), which is not further metabolized during the early stages of ripening, resulting in a higher pH at the time of analysis. Residual galactose may be metabolized by NSLAB during further ripening resulting in a subsequent decrease in pH as ripening progresses (Turner, Morris, & Martley, 1983). In a regular Swiss style system, *L. helveticus* and increasing NSLAB populations utilise residual galactose to form D- and L-lactate (Fox, Lucey, & Cogan, 1990; McSweeney & Fox, 2004).

3.1.2. Chemical composition of inner and outer layers

Levels of salt and S/M were significantly higher in the outer layers in comparison to the cheese interior, as expected (Table 2). Similarly, levels of FDM, moisture and MNFS were significantly higher ($P < 0.05$) in the inside compared to the outside layers. Lower moisture levels in the outside layer of cheeses are due to the movement of Na^+ and Cl^- ions from the brine into the cheese matrix with the subsequent expulsion of moisture from the outer most layer of the cheese in order to restore the resulting osmotic pressure balance as described by Guinee and Fox (2004). However, there was no significant difference in pH between the outside and inside layers for all cheeses, and fat levels were similar for all treatments both in the outside and inside layer of the cheeses. Protein levels were similar in both layers of cheeses made with *S. thermophilus* only and with starters in combination (regardless of brine concentration) while cheese made using *L. helveticus* only had significantly higher ($P < 0.05$) protein levels in the outside layer of cheese compared to the inside layer.

3.2 Viability of starter and NSLAB populations

3.2.1 *Lactobacillus helveticus*

In cheeses manufactured with *L. helveticus*, mean viable counts reached $\geq 10^{7.5}$ cfu/g⁻¹ (Fig. 1 A) prior to brining irrespective of the inoculation level used. These counts are similar to those observed for Swiss- type cheese using similar cultures (Sheehan, et al., 2007; 2008). A slight decline in cell numbers was evident in cheeses using a combination starter blend after 66 hours of brining for both the outside and inside layers of the cheese. A significant decline ($P < 0.01$) in cell viability was evident in cheese made using *L. helveticus* only from 0 d onwards with no viable cells present from 7 d of ripening onwards in the high salt outside layer. Cell counts in the inside layer of all cheeses containing *L. helveticus* showed a visual decline over the 26 d ripening period although not statistically significant ($P > 0.05$). . *L.*

helveticus viability in the outside layer of cheese made using both starters in combination and brined at 22 % declined significantly ($P < 0.01$) from 4 d of ripening onwards with no viable cells evident after 18 d of ripening. The inside layer showed a much slower decline in cell viability with significantly higher counts compared to the outside layer from 18 d of ripening onwards ($P < 0.05$) with final cell counts of 10^5 cfu/g⁻¹ in the inside layer at 26 d of ripening. Cheese manufactured with combined starters and brined at 18 % had significantly lower levels of cell viability in the outside, compared to the inside layer ($P < 0.05$) of the cheeses throughout ripening, and similarly but to a lesser degree for the cheeses brined at 22 %.

3.2.2 *Streptococcus thermophilus*

For cheese manufactured with *S. thermophilus* mean viable bacterial counts reached $\geq 10^{8.5}$ cfu/g⁻¹ before brining in ST, ST/LH 22 % and ST/LH 18 % cheeses and remained at those counts after the 66hrs of brining in the outside layer of cheeses. No significant decrease in *S. thermophilus* viability ($P > 0.05$) was evident in the outside layer of any cheese over the 26 day sampling period. A slight, although non-significant ($P > 0.05$), decrease in *S. thermophilus* viability was observed within the inside layer of cheeses with counts, after brining, of 10^8 cfu/g⁻¹ decreasing to 10^7 and $10^{7.5}$ cfu/g⁻¹ respectively in the two combination cheeses ST/LH 22 % and ST/LH 18 %. *S. thermophilus* TH3 has an indicated salt sensitivity of 50 % inhibition at 2.1 % NaCl and 100 % inhibition at > 4 % NaCl (manufacturer's specification). Milci, Goncu, AlpKent, and Yaygin (2005) looked at the effect of salt on microbiological populations in Halloumi cheese and found that in cheese with a salt concentration of > 4 % NaCl, there was no significant difference in *S. thermophilus* viability over the first 30 days of ripening indicating that *S. thermophilus* is capable of maintaining high viability counts during the early stages of ripening in high salt conditions, similar to that observed in this study.

3.2.3 NSLAB

NSLAB are adventitious microbiota which contribute a large portion of the overall microbiota in most ripening cheeses. They are mostly mesophilic lactobacilli and pediococci and can result from post production contamination or from the pasteurized milk itself due to incomplete inactivation. NSLAB populations traditionally start out at very low levels during early ripening, and gradually increase as the starter populations decline, and are usually the predominant microbiota at the completion of ripening (Sheehan, et al., 2007; 2008). In this study we noted significant differences in mean viable NSLAB counts between the high salt outside layer and low salt inside layer of the experimental cheeses. Cheeses made with *S. thermophilus* only, or with a combination of *S. thermophilus* and *L. helveticus* (ST/LH 22 %, ST/LH 18 %), had significantly higher counts ($P < 0.05$) of NSLAB bacteria in the inside layer compared to the outside layer of cheeses from 7 d to 18 d of ripening. After 18 days there was a significant increase in the NSLAB population on the outside of the *S. thermophilus* only and the ST/LH 18 % cheeses. Final NSLAB counts of $10^{5.5-6.1}$ cfu/g⁻¹ after 26 d of ripening in the inside layer of cheeses are similar to those reported by Sheehan, et al. (2007); (2008) for similar style cheeses of a similar age. The absence of salt in the inside layer is the most likely cause for the higher NSLAB numbers, while high S/M levels of > 6-8 % as observed in the outside layers have been shown to retard NSLAB growth especially in the early stages of ripening (Guinee & Fox, 2004; Jordan & Cogan, 1993).

3.3 Lactate dehydrogenase activity

Lactate dehydrogenase is an intracellular enzyme found in many lactic acid bacteria including *L. helveticus* and NSLAB, and is used as a marker for cell lysis in dairy products especially cheese (Collins, McSweeney, & Wilkinson, 2003; Crow, et al., 1995; Hannon, et al., 2003; Kenny, FitzGerald, O'Cuinn, Beresford, & Jordan, 2006; Sheehan, et al., 2006). In

this study, significant differences ($P < 0.05$) were seen in LDH activity between the outside and inside sections of the ripening cheeses indicating a significant effect of salt. In all cheeses containing *L. helveticus*, significantly higher LDH activity ($P < 0.05$) was observed in the inside layer compared to the outside layer of cheese (Fig. 2). There was a significant increase in LDH activity observed in all cheeses from pre-brine to 0 d of ripening, possibly due to the rapid cooling of the cheese curd on placing into the brine solution ($\sim 12^\circ\text{C}$).

Previous studies have indicated varying levels of enzyme inhibition due to salt and S/M levels. Vafopoulou-Mastrogiannaki (1999) found that increasing S/M levels from 3 to 6 % significantly reduced the activity of intracellular aminopeptidases. Gobbetti, et al. (1999b) investigated the influence of pH (5.5-5.7) and S/M levels (0.0-7.5% w/w) on proteolytic activity of starter and NSLAB and results indicated a relative insensitivity of peptidases produced by *Lb. casei* subsp. *casei* and *Lb. plantarum* to both pH and salt levels in the ranges studied. The effect of salt on enzyme activity is both enzyme- and species-specific (Gobbetti, et al., 1999a). The high level of S/M observed in the outside layer for this study at 0 d of ripening ($> 11\%$) rapidly declines to much lower levels of $< 7\%$ by 11 d of ripening, suggesting inhibition of intracellular enzymes throughout ripening due to salt levels alone is unlikely.

The highest levels of LDH activity were observed in the inside layer of cheeses made using a combination of *S. thermophilus* and *L. helveticus* with both 22% and 18% brine concentrations. These cheeses showed LDH activity levels of > 7.0 LDH units/ ml of sample compared to < 4.0 LDH units/ml of sample in the corresponding outside layers after 11 d of ripening. These results also suggest that cell lysis and enzyme release is not only salt dependent and many factors can influence the viability of starter bacterial cells including

These results combined with the viable cell counts for *L. helveticus* could suggest that bacterial cells are becoming non culturable due to the high levels of salt and resultant osmotic stress present in the outside layer of cheeses, but this is not corresponding to an increase in enzymatic activity. A possible reason for this is, *L. helveticus* cells may be entering into a survival state due to initial heat shock due to super-optimal temperatures associated with the max scald temperature used (50 °C) in combination with subsequent high salt concentration, possibly resulting in survival mechanisms such as, rearrangement of fatty acids in the bacterial cell membrane (Guerzoni, Lanciotti, & Cocconcelli, 2001). This could result in the membrane becoming less porous and increasingly rigid, thus preventing the entrance of sodium ions and also preventing the release of intracellular enzymes, as seen in previous studies on *Lactobacilli* bacteria in milk whey and sea water respectively (Guerzoni, Lanciotti, & Cocconcelli, 2001; Palomino, Allievi, Gründling, Sanchez-Rivas, & Ruzal, 2013). To fully determine the fate of salt on the bacterial cell membrane further studies are needed to specifically characterise the reaction of the bacterial membrane to exposure to salt levels similar to those observed in this study.

3.4 Flow cytometry

3.4.1 Non-invasive sample preparation

FC analysis of the microbiota of milk and dairy products requires the development of sample preparation protocols aimed at minimizing levels of milk proteins and lipid particles, which can interfere with bacterial staining and detection (Doolan, Wilkinson, & Hickey, 2015). Incubation with proteinases has been proposed as an approach to overcome these issues (Doherty, et al., 2010; Gunasekera, Attfield, & Veal, 2000). Such treatment was avoided in this study as it could potentially trigger ROS generation and membrane

permeabilization in bacterial cells, thereby altering the assessment of oxidative stress and membrane integrity by FC. A novel, enzyme-free protocol was utilized, which featured two sequential separations of the microbiota from the large particles of the cheese homogenate by means of filter bags of decreasing pore sizes (280 μm and 63 μm) and the replacement of phosphate buffer saline with MRD as sample and dilution buffer to maximize cell recovery. This fast, simple and non-invasive protocol consistently delivered a cheese bacterial extract of good quality for FC analysis, exhibiting minimal cell aggregation and interference from proteins and lipids (supplementary Fig. 1).

3.4.2 Cell membrane integrity

Membrane integrity is one of the predominant indicators of cell viability and can be measured by FC following a classical double stain approach leveraging the different membrane permeation characteristics of a “dead” dye (e.g. PI) and a “live” counterstain (e.g. Syto 9) (Cronin, 2015). While both dyes non-specifically stain any bacterium, the populations of live (Syto 9⁺/PI⁻) *S. thermophilus* and *L. helveticus* starters were found to be clearly discriminated on density biplots of Syto 9 vs PI fluorescence since the pre-brining stage (Figure 3). The *L. helveticus* population exhibited larger fluorescence intensities for both dyes than the *S. thermophilus* population on the biplots of single starter cheeses (Figure 3 A,B) and the two microbial groups maintained the same relative positioning when present together in the cheese (Figure 3 C,D). This behaviour is attributed to the significantly different morphologies of the two starters, as the long rod-shaped *L. helveticus* may accumulate more dye molecules and consequently emit larger fluorescence than the small coccoids of *S. thermophilus*. This finding also enabled the use of gating strategies to specifically monitor the viability of each starter from the pre-brining stage up to 26 d of ripening (Figure 3 C,D). This

approach presented some limitations such as the partial overlapping between the populations of live *L. helveticus* and injured cells as well as the potential overlap with the forthcoming non-starter flora. However, in absence of species-specific probes and considering the short ripening time of this study, we pursued the intriguing prospect to assess the impact of differential salt levels on the membrane status of each starter.

Figure 4 shows the temporal dynamics of the live cell counts of gate 1 (or potential *S. thermophilus*) population, gate 2 (or potential *L. helveticus*) population and combined gates 1 and 2 (or combined starters) populations before, during and up to 26 d after brining. Compared to plate counts, the FC approach returned higher *L. helveticus* counts, especially in the cheese made with *S. thermophilus* only, whereas the FC counts of viable *S. thermophilus* closely matched those obtained by the traditional plate count method. The viability of both individual and combined starters declined significantly faster in the core ($P < 0.05$) compared to the surface of all ripening cheeses. This trend appears to be in agreement with the microbial lysis data showing larger release of intracellular enzymes in the inner compared to the outer cheese layers (Fig. 2), but failed to explain the discrepancy with the plate counts indicating higher bacterial survival in the core of the cheeses (Figure 1 A,B).

3.4.3 Effect of salt on morphology of starter bacteria

In this study, brining was found to significantly impact the morphology of the microbiota located in the outer layers of the cheeses as a reduction in cell size after 18 h ($P < 0.01$) and 48 h ($P < 0.001$) of brining compared to pre-brine values was observed (Figure 5B). Temporal analysis of the median FSC values revealed that the microbiota residing in the cheeses outer layers experienced a continuous and significant decrease ($P < 0.01$) in cell size upon brining and across the first 26 d of ripening, whereas the size of the microorganisms

located in the cheese cores remained substantially unaltered ($P > 0.05$) (Figure 5A & C). When confronted with osmotic shifts, bacteria are capable of activating channels and transporters to rapidly adjust the intracellular osmolyte pools in proportion to the osmotic stress. In this study, the S/M content of the cheeses' outer layers was on average around 8 % and 6-fold higher than that of the inner layers immediately after brining. It is therefore reasonable to assume that such a large hyperosmotic pressure could have exceeded cells' capacity to counteract it thereby causing water efflux and volume reduction (Poolman, Spitzer, & Wood, 2004). Concentration of the cytoplasm disrupts cell structure and metabolic functionality (Poolman, et al., 2004), without necessarily compromising membrane integrity. On the contrary, modifications in membrane composition and rigidity are known mechanisms of bacterial survival triggered by osmotic stress as discussed previously. We therefore postulate that the high salinity environment of the cheese outer layer may have impacted microbial physiology by causing cell shrinkage and possibly increasing cell membrane rigidity. This dual effect could explain the apparent contradiction between the results of plate counts, showing lowest microbial viability in the cheese outer layers, and the results of the FC and microbial lysis assays, suggesting lowest survival in the cheese cores.

Additionally, FC and microscopy imaging have shown that prolonged hyperosmotic stress induces condensation of the nucleoid with association of DNA and RNA polymerase molecules leading to the emergence of multiple subpopulations in bacteria (Cagliero & Jin, 2012; Sachidanandham & Gin, 2009). A similar phenomenon was observed in the Syto 9 vs PI biplots of the microbiota located in the cheese outer layer as a distinct small population with lower Syto 9 intensity emerged beside the main *S. thermophilus* population during brining (Figure 6 A). This secondary population was not observed in the outer layer samples and in all probability represented the most osmotically stressed portion of the *S. thermophilus* population, providing further evidence of the significant impact that high salt environments,

such as in the outer layer of brine salted cheeses, may exert on the physiology and viability of the local microbiota.

3.4.4 Effect of salt concentration on oxidative stress of starter bacteria

The cell-permeable, ROS-sensitive CRG probe was used in combination with FC to investigate the differential impact exerted by the hypo and hyperosmotic environments of the inner and outer cheese layers, respectively, on the oxidative stress status of the local microbiota. Temporal analysis of the CRG fluorescence intensity showed the highest levels to be present in pre-brine samples (Figure 7) indicating that cheese manufacturing exerted a significant oxidative stress resulting in large accumulation of free ROS in the cheese microbiota. Brining added further oxidative stress to the microbial cells as the significant drop ($P < 0.05$) in CRG fluorescence seen after brining (0 d) suggested reaction with and oxidation of cellular targets by the previously accumulated free ROS, which therefore went undetected by the CRG probe. This turnover of free and bound ROS molecules continued, although at much lower rates, in all cheeses for the first 7 d of ripening. A significant accumulation ($P < 0.01$) of ROS molecules was detected in the cheeses made with only *L. helveticus* and with both starters from 11 d of ripening onwards, whereas the cheese made with only *S. thermophilus* showed a significant rise ($P < 0.01$) in ROS levels only after 18 d of ripening. This suggests that the cheese microbiota experiences situations of oxidative stress at various stages of ripening, possibly in relation to times of enhanced competition for nutrients deriving from the progressive rise of the NSLAB population (Fig. 1 C).

Bacterial cells located in the cheese surface were found to be more subjected to oxidative stress than those of the cheese core within LH only and combined starter cheeses, as significantly higher levels ($P < 0.05$) of free ROS were detected in the outer layer in most of

the samples. Larger ROS levels correlate with a larger potential for oxidative damage, therefore these findings confirm that the cheese microbiota is affected differentially by the large gradients of salt typical of very young brined cheeses and also provides the physiological basis supporting the lowest plate counts found in the cheese surface (Figure 1 A). In the *S. thermophilus*-only cheese, higher ROS levels were mostly detected in the cheese core but, after 26 d of ripening, larger amounts of ROS were again found in the cheese surface. The CRG data was in agreement with the plate counts showing larger decline of *S. thermophilus* viability in the cheese inner layer (Fig. 1 B).

3.5 Proteolysis during cheese ripening

3.5.1 Primary proteolysis

Primary proteolysis is an indication of the initial breakdown of α_{s1} -casein and β -casein molecules during cheese manufacture and ripening (Sheehan, et al., 2008; Upadhyay, McSweeney, Magboul, & Fox, 2004). Breakdown is linked mostly to naturally present plasmin/ plasminogen in milk and the retention of residual coagulant used during manufacture, which in this case, is the highly proteolytic and thermolabile (readily inactivated at temperatures $> 48^{\circ}\text{C}$) enzyme endothiapepsin produced by the fungus *Cryphonectria parasitica* (Garnot & Molle, 1987). Endothiapepsin has similar proteolytic properties to Pepsin A and readily targets β -casein forming variants of γ -casein and also breaks down α_{s1} -casein but at a slower rate compared to enzymes such as chymosin (Awad, Lüthi-Peng, & Puhani, 1999).

The results observed in this study indicate a significant increase ($P < 0.05$) in levels of primary proteolysis for all cheeses over the 26 d of ripening except in the case of the outer layer of *S. thermophilus*-only cheese. The increase in primary proteolysis may be due to potential activation of native plasminogen due to the high cook temperature associated with

Swiss type cheese (Somers & Kelly, 2002) and possible activity of the aspartic indigenous proteinase cathepsin D (Sousa, Ardö, & McSweeney, 2001), resulting in increased breakdown of the β - and α_{s1} - caseins. Levels of % pH 4.6 SN/TN were significantly higher ($P < 0.05$) in the inside layer compared to the outside layer of all cheeses. (Fig. 8 A). The breakdown of major proteins visible after 26 d of ripening is minimal and therefore the differences observed within a Urea-PAGE gel performed at 26 d of ripening are not pronounced. However, a trend was observed where β - casein, and α_{s1} - casein to a lesser extent, appear to be more degraded in the inside layers of the cheeses compared to the outside layer, as indicated by an increase in the intensity for bands associated with γ_1 and γ_3 - CN and α_{s1} - CN (f102-199) resulting from the degradation of β - and α_{s1} - CN. The largest breakdown appears to be of β - CN in the inside layer of both cheeses made with starters in combination which is in agreement with levels of pH4.6SN/TN. This would indicate reduced breakdown of caseins and a retarded activity of native proteolytic enzymes in the higher salt outside layers (supplementary material).

Increasing salt concentration has been shown to increase protein hydration and promote casein solubilisation which may bind with Na^+ to form a casein/Na complex (Guinee & Fox, 2004; Pastorino, Hansen, & McMahon, 2003). These casein/Na complexes are more difficult for the proteolytic enzymes to actively break down and combined with the possible inhibition of proteolytic enzymes due to the high salt environment may lead to reduced levels of primary proteolysis like those observed in this study.

3.5.2 Secondary proteolysis

The levels of Free Amino Acids (FAA's) present within the cheese throughout ripening is a measure of further breakdown of casein molecules and fragments, resulting from the

primary stage of proteolysis, by both extracellular and intracellular proteolytic enzymes released by the microbiota present. Autolytic bacteria such as mesophilic lactococci and thermophilic lactobacilli (*L. helveticus*) have been linked to increased levels of secondary proteolysis as increasing levels of FAA's correspond with increased enzymatic activity resulting from starter culture cell lysis. Levels of total FAA's increased significantly ($P < 0.01$) during ripening in both the outside and inside layers of all cheeses (except cheese made using *S. thermophilus* only. Significantly higher levels of FAA's ($P < 0.05$) were detected in the inside layer compared to the outside layer for all cheeses (except the *S. thermophilus* only cheese at 26 d of ripening). Cheese made using *L. helveticus* only had the highest levels of FAA's in both the inside and outside layers in comparison to their respective counterparts in all other cheeses (Fig. 8 B).

The impact of salt concentration and starter type on individual FAA levels at 26 d of ripening are shown Table 3. Significant levels of glutamate, leucine and lysine and elevated levels of alanine, valine, arginine and proline were evident in cheeses made using *L. helveticus* corresponding to a typical FAA profile of Swiss- style cheese during the early stages of ripening (Lawlor, Delahunty, Wilkinson, & Sheehan, 2002; Sheehan, et al., 2007; Sheehan, et al., 2008). The inside layer of cheeses manufactured using *L. helveticus*- only, or in combination, contained significantly higher levels of glutamate, alanine, valine, leucine, lysine, arginine and proline compared to the corresponding outside layer after 26 d of ripening, which is in agreement with LDH enzymatic results (Fig. 2).. Cheese made using *S. thermophilus*-only did not contain significant levels of any FAA's and it can therefore be suggested that it is not a significant contributor to secondary proteolysis in cheese.

4. Conclusion

In this study, a multi-disciplinary approach leveraging conventional microbiology, biochemistry and flow cytometry methods was used to gain a deeper understanding of the differential impact exerted by the low and high salt environments of the cheese inner and outer layers, respectively, on the viability and physiology of the starter microbiota in brine salted cheeses. Results obtained via traditional plate counts were contradictory when compared with results for enzyme activity and release of free amino acid and with results obtained by FC. High salt concentration resulted in reduced primary and secondary proteolysis in the outside layer of cheeses which could significantly impact on ripening homogeneity in brine salted cheeses resulting in varied amino acid profiles and volatile components through the cheese block.

Analysis by FC showed microbial cell shrinkage in the cheese outer layers, which was not observed in the cheese core and which supported the hypothesis that starter bacteria may undergo membrane physiological changes to survive the high saline environment, possibly resulting in a more rigid bacterial cell membrane, and potentially promoting loss of culturability.

The detection of larger levels of ROS in the microbiota of the cheese outer layer reflected a probable higher oxidative stress due to the high salt environment and, consequently, to a larger potential for accumulated ROS to cause cell death. Trends observed by FC provided a physiological basis to explain observed contradictions between lower viable cell counts and reduced LDH release and membrane damage in the cheese outer layer. It is proposed that cells in the cheese outer layer could have died as a consequence of their inability to scavenge the excess levels of ROS generated and to prevent the oxidative damage of critical cellular functions. In our opinion, this dual mechanism of cell death explains the gap observed between results of viable cell counts, enzyme activity and membrane integrity.

The overall results of this study challenge the current assumptions regarding the role of salt in controlling bacterial growth. The concept that bacterial growth is reduced by salt due to cell lysis, resulting in enzyme release, may no longer be valid in relation to brine salted cheese types, where large variations in salt concentration exist immediately after brining. The reduction in levels of primary and secondary proteolysis in the outside layer of cheeses could have a significant impact on the ripening homogeneity in any brine salted cheese, causing potential quality and consumer acceptability issues.

The results of this research forces cheese manufacturers to reconsider how they view salt and brine salting specifically in relation to control of starter bacteria populations. These results may also redefine the pattern of proteolysis and corresponding texture and flavour development throughout the ripening cheese block/ wheel. It also poses questions as to ways of exploiting this new information in order to influence ripening patterns or to increase ripening intensity and the possibility of creating high quality reduced sodium cheeses via brine salting and starter strain selection. The current research findings could be harnessed by the global cheese industry in a time where milk supply and cheese production is increasing and where cheese manufacturers are looking for ways to diversify their product ranges and increase economic return.

Acknowledgment

This research was funded by the Dairy Levy Trust, Ireland.

Bibliography

- Andrews, A. T. (1983). Proteinases in normal bovine milk and their action on caseins. *Journal of Dairy Research*, 50, 45-55.
- Awad, S., Lüthi-Peng, Q.-Q., & Puhani, Z. (1999). Proteolytic Activities of Suparen and Rennilase on Buffalo, Cow, and Goat Whole Casein and β -Casein. *Journal of Agricultural and Food Chemistry*, 47, 3632-3639.
- Blakesley, R. W., & Boezi, J. A. (1977). A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. *Analytical Biochemistry*, 82, 2.
- Bunthof, C. J., van Schalkwijk, S., Meijer, W., Abee, T., & Hugenholtz, J. (2001). Fluorescent method for monitoring cheese starter permeabilization and lysis. *Applied and environmental microbiology*, 67, 4264-4271.
- Bunthof, C. J., & Abee, T. (2002). Development of a flow cytometric method to analyze subpopulations of bacteria in probiotic products and dairy starters. *Applied and environmental microbiology*, 68, 2934-2942.
- Cagliero, C., & Jin, D. J. (2012). Dissociation and re-association of RNA polymerase with DNA during osmotic stress response in Escherichia coli. *Nucleic acids research*, gks988.
- Choi, H., Yang, Z., & Weisshaar, J. C. (2015). Single-cell, real-time detection of oxidative stress induced in Escherichia coli by the antimicrobial peptide CM15. *Proceedings of the National Academy of Sciences of the United States of America*, 112, E303-E310.
- Cogan, T. M., O'Dowd, M., & Mellerick, D. (1981). Effects of pH and Sugar on Acetoin Production from Citrate by *Leuconostoc lactis*. *Applied and environmental microbiology*, 41, 1-8.
- Collins, Y. F., McSweeney, P. L. H., & Wilkinson, M. G. (2003). Evidence of a relationship between autolysis of starter bacteria and lipolysis in Cheddar cheese during ripening. *Journal of Dairy Research*, 70, 105-113.
- Comas-Riu, J., & Rius, N. (2009). Flow cytometry applications in the food industry. *Journal of industrial microbiology & biotechnology*, 36, 999-1011.
- Cronin, U. P. (2015). The Use of Flow Cytometry to Study Sporeforming Bacteria. In M. G. Wilkinson (Ed.), *Flow Cytometry In Microbiology: Technology and Applications*. (pp. 109-118). UK: Caister Academic Press.
- Crow, V. L., Coolbear, T., Gopal, P. K., Martley, F. G., McKay, L. L., & Riepe, H. (1995). The role of autolysis of lactic acid bacteria in the ripening of cheese. *International Dairy Journal*, 5, 855-875.
- den Besten, H. M., Mols, M., Moezelaar, R., Zwietering, M. H., & Abee, T. (2009). Phenotypic and transcriptomic analyses of mildly and severely salt-stressed *Bacillus cereus* ATCC 14579 cells. *Applied and environmental microbiology*, 75, 4111-4119.
- Díaz, M., Herrero, M., García, L. A., & Quirós, C. (2010). Application of flow cytometry to industrial microbial bioprocesses. *Biochemical engineering journal*, 48, 385-407.
- Doherty, S., Gee, V., Ross, R., Stanton, C., Fitzgerald, G., & Brodkorb, A. (2010). Efficacy of whey protein gel networks as potential viability-enhancing scaffolds for cell immobilization of *Lactobacillus rhamnosus* GG. *Journal of microbiological methods*, 80, 231-241.
- Doolan, I. A., & Wilkinson, M. G. (2009). Comparison of the effects of various attenuation methods on cell permeability and accessibility of intracellular enzymes in *Lactococcus lactis* strains. *International Dairy Journal*, 19, 215-221.
- Doolan, I. A., Nongonierma, A. B., Kilcawley, K. N., & Wilkinson, M. G. (2014). Partitioning of starter bacteria and added exogenous enzyme activities between curd and whey during Cheddar cheese manufacture. *International Dairy Journal*, 34, 159-166.

- Doolan, I. A., Wilkinson, M. G., & Hickey, D. K. (2015). The Application of Flow Cytometry to the Study of Lactic Acid Bacteria Fermentations. In M. G. Wilkinson (Ed.), *Flow Cytometry In Microbiology: Technology and Applications* (pp. 159-173). UK: Caister Academic Press.
- Fenelon, M., O'Connor, P., & Guinee, T. (2000). The effect of fat content on the microbiology and proteolysis in Cheddar cheese during ripening. *Journal of Dairy Science*, 83, 2173-2183.
- Floury, J., Jeanson, S., Aly, S., & Lortal, S. (2010). Determination of the diffusion coefficients of small solutes in cheese: A review. *Dairy Science & Technology*, 90, 477-508.
- Floury, J., Madec, M. N., Waharte, F., Jeanson, S., & Lortal, S. (2012). First assessment of diffusion coefficients in model cheese by fluorescence recovery after photobleaching (FRAP). *Food Chemistry*, 133, 551-556.
- Fox, P., Lucey, J., & Cogan, T. (1990). Glycolysis and related reactions during cheese manufacture and ripening. *Critical Reviews in Food Science & Nutrition*, 29, 237-253.
- Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2000). Salting of cheese curd. In *Fundamentals of cheese science* (Vol. 1, pp. 153-168). Gaithersburg, Maryland: Aspen Publishers, Inc.
- Garnot, P., & Molle, D. (1987). Heat-Stability of Milk-Clotting Enzymes in Conditions Encountered in Swiss Cheese Making. *Journal of Food Science*, 52, 75-77.
- Gobbetti, M., Lanciotti, R., De Angelis, M., Corbo, M. R., Massini, R., & Fox, P. (1999a). Study of the effects of temperature, pH, NaCl, and a_w on the proteolytic and lipolytic activities of cheese-related lactic acid bacteria by quadratic response surface methodology. *Enzyme and Microbial Technology*, 25, 795-809.
- Gobbetti, M., Lanciotti, R., De Angelis, M., Corbo, M. R., Massini, R., & Fox, P. F. (1999b). Study of the effects of temperature, pH and NaCl on the peptidase activities of non-starter lactic acid bacteria (NSLAB) by quadratic response surface methodology. *International Dairy Journal*, 9, 865-875.
- Gomes, A. M. P., Vieira, M. M., & Malcata, F. X. (1998). Survival of probiotic microbial strains in a cheese matrix during ripening: Simulation of rates of salt diffusion and microorganism survival. *Journal of Food Engineering*, 36, 281-301.
- Guerzoni, M. E., Lanciotti, R., & Cocconcetti, P. S. (2001). Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*. *Microbiology*, 147, 2255-2264.
- Guinee, T. (2004). Salting and the role of salt in cheese. *International Journal of Dairy Technology*, 57, 99-109.
- Guinee, T. P., & Fox, P. F. (2004). Salt in Cheese: Physical, Chemical and Biological Aspects. In P. L. H. M. T. M. C. Patrick F. Fox & P. G. Timothy (Eds.), *Cheese: chemistry, physics and microbiology* (Vol. Volume 1, pp. 207-259): Academic Press.
- Gunasekera, T. S., Attfield, P. V., & Veal, D. A. (2000). A flow cytometry method for rapid detection and enumeration of total bacteria in milk. *Applied and environmental microbiology*, 66, 1228-1232.
- Hannon, J., Wilkinson, M., Delahunty, C., Wallace, J., Morrissey, P., & Beresford, T. (2003). Use of autolytic starter systems to accelerate the ripening of Cheddar cheese. *International Dairy Journal*, 13, 313-323.
- Hickey, D. K., Kilcawley, K. N., Beresford, T. P., Sheehan, E. M., & Wilkinson, M. G. (2007). Starter strain related effects on the biochemical and sensory properties of Cheddar cheese. *Journal of Dairy Research*, 74, 9-17.

- IDF. (1988a). *Estimation of thermophilic lactobacilli*. In (Vol. International standard 117a). Brussels, Belgium International Dairy Federation.
- IDF. (1988b). Cheese and processed cheese: Determination of chloride content (potentiometric titration method). In (Vol. International Standard 88a). Brussels, Belgium: International Dairy Federation.
- IDF. (1993). *Milk: Determination of the nitrogen content (Kjeldahl method) and calculation of crude protein content*. In (Vol. International Standard 20b). Brussels, Belgium: International Dairy Federation.
- Jordan, K., & Cogan, T. (1993). Identification and growth of non-starter lactic acid bacteria in Irish Cheddar cheese. *Irish Journal of Agricultural and Food Research*, 47-55.
- Kenny, O., FitzGerald, R. J., O’Cuinn, G., Beresford, T., & Jordan, K. (2006). Autolysis of selected *Lactobacillus helveticus* adjunct strains during Cheddar cheese ripening. *International Dairy Journal*, 16, 797-804.
- Kuchroo, C., & Fox, P. (1982). Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. *Milchwissenschaft. = Milk science international*.
- Lawlor, J. B., Delahunty, C. M., Wilkinson, M. G., & Sheehan, J. (2002). Relationships between the gross, non-volatile and volatile compositions and the sensory attributes of eight hard-type cheeses. *International Dairy Journal*, 12, 493-509.
- Lee, H. J., Olson, N. F., & Lund, D. B. (1980). Diffusion of Salt, Fatty Acids, and Esterases in Mozzarella Cheese. *Journal of Dairy Science*, 63, 513-522.
- McSweeney, P. L. H., & Fox, P. F. (2004). Metabolism of residual lactose and of lactate and citrate. In P. L. H. M. T. M. C. Patrick F. Fox & P. G. Timothy (Eds.), *Cheese: chemistry, physics and microbiology* (Vol. Volume 1, pp. 361-371): Academic Press.
- Milci, S., Goncu, A., AlpKent, Z., & Yaygin, H. (2005). Chemical, microbiological and sensory characterization of Halloumi cheese produced from ovine, caprine and bovine milk. *International Dairy Journal*, 15, 625-630.
- Pajonk, A. S., Saurel, R., & Andrieu, J. (2003). Experimental study and modeling of effective NaCl diffusion coefficients values during Emmental cheese brining. *Journal of Food Engineering*, 60, 307-313.
- Palomino, M. M., Allievi, M. C., Gründling, A., Sanchez-Rivas, C., & Ruzal, S. M. (2013). Osmotic stress adaptation in *Lactobacillus casei* BL23 leads to structural changes in the cell wall polymer lipoteichoic acid. *Microbiology*, 159, 2416-2426.
- Pastorino, A. J., Hansen, C. L., & McMahon, D. J. (2003). Effect of Salt on Structure-Function Relationships of Cheese1. *Journal of Dairy Science*, 86, 60-69.
- Payne, M. R., & Morison, K. R. (1999). A multi-component approach to salt and water diffusion in cheese. *International Dairy Journal*, 9, 887-894.
- Poolman, B., Spitzer, J. J., & Wood, J. M. (2004). Bacterial osmosensing: roles of membrane structure and electrostatics in lipid-protein and protein-protein interactions. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1666, 88-104.
- Rogosa, M., Mitchell, J. A., & Wiseman, R. F. (1951). A selective medium for the isolation and enumeration of oral and fecal lactobacilli. *Journal of bacteriology*, 62, 132.
- Rulikowska, A., Kilcawley, K. N., Doolan, I. A., Alonso-Gomez, M., Nongonierma, A. B., Hannon, J. A., & Wilkinson, M. G. (2013). The impact of reduced sodium chloride content on Cheddar cheese quality. *International Dairy Journal*, 28, 45-55.
- Sachidanandham, R., & Gin, K. Y.-H. (2009). Flow cytometric analysis of prolonged stress-dependent heterogeneity in bacterial cells. *FEMS Microbiology Letters*, 290, 143-148.
- SAS. (1995). *SASs user’s guide: statistics (version 6.12 edition)*. Cary, NC, USA: SAS institute.
- Scott, R. (1981). *Selected cheese recipes*. Essex, London, UK: Applied Science Publishers Ltd.

- Sheehan, A., O'Loughlin, C., O'Cuinn, G., FitzGerald, R. J., & Wilkinson, M. G. (2005). Cheddar cheese cooking temperature induces differential lactococcal cell permeabilization and autolytic responses as detected by flow cytometry: implications for intracellular enzyme accessibility. *Journal of Applied Microbiology*, 99, 1007-1018.
- Sheehan, A., Cuinn, G. O., FitzGerald, R. J., & Wilkinson, M. G. (2006). Proteolytic enzyme activities in Cheddar cheese juice made using lactococcal starters of differing autolytic properties. *Journal of Applied Microbiology*, 100, 893-901.
- Sheehan, A., O'Cuinn, G., FitzGerald, R. J., & Wilkinson, M. G. (2009). Distribution of microbial flora, intracellular enzymes and compositional indices throughout a 12kg Cheddar cheese block during ripening. *International Dairy Journal*, 19, 321-329.
- Sheehan, J. J., Fenelon, M. A., Wilkinson, M. G., & McSweeney, P. L. H. (2007). Effect of cook temperature on starter and non-starter lactic acid bacteria viability, cheese composition and ripening indices of a semi-hard cheese manufactured using thermophilic cultures. *International Dairy Journal*, 17, 704-716.
- Sheehan, J. J., Wilkinson, M. G., & McSweeney, P. L. H. (2008). Influence of processing and ripening parameters on starter, non-starter and propionic acid bacteria and on the ripening characteristics of semi-hard cheeses. *International Dairy Journal*, 18, 905-917.
- Somers, J. M., & Kelly, A. L. (2002). Contribution of plasmin to primary proteolysis during ripening of cheese: effect of milk heat treatment and cheese cooking temperature. *Lait*, 82, 181-191.
- Sousa, M. J., Ardö, Y., & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal*, 11, 327-345.
- Sutherland, B. J. (2002). Salting of cheese. In H. Roginski, J. W. Fuquay & P. F. Fox (Eds.), *Encyclopedia of Dairy Sciences* (pp. 293-300). London: Academic Press.
- Terzaghi, B. E., & Sandine, W. (1975). Improved medium for lactic streptococci and their bacteriophages. *Applied microbiology*, 29, 807-813.
- Thomas, T. D., & Pearce, K. N. (1981). Influence of salt on lactose fermentation and proteolysis in Cheddar cheese. *New Zealand Journal of Dairy Science and Technology*, 16, 7.
- Tsuzuki, M., Moskvina, O. V., Kuribayashi, M., Sato, K., Retamal, S., Abo, M., Zeilstra-Ryalls, J., & Gomelsky, M. (2011). Salt stress-induced changes in the transcriptome, compatible solutes, and membrane lipids in the facultatively phototrophic bacterium *Rhodobacter sphaeroides*. *Applied and environmental microbiology*, 77, 7551-7559.
- Turhan, M., & Kaletunc, G. (1992). Modeling of Salt Diffusion in White Cheese during Long-Term Brining. *Journal of Food Science*, 57, 1082-1085.
- Turhan, M., & Gunasekaran, S. (1999). Analysis of moisture transfer in White cheese during brining. *Milchwissenschaft*, 54, 446-450.
- Turner, K. W., Morris, H. A., & Martley, F. G. (1983). Swiss-type cheese. II. The role of thermophilic lactobacilli in sugar fermentation. *New Zealand Journal of Dairy Science and Technology*.
- Upadhyay, V. K., McSweeney, P. L. H., Magboul, A. A. A., & Fox, P. F. (2004). Proteolysis in cheese during ripening. In P. L. H. M. T. M. C. Patrick F. Fox & P. G. Timothy (Eds.), *Cheese: chemistry, physics and microbiology* (Vol. Volume 1, pp. 391-VIII): Academic Press.
- Vafopoulou-Mastrogiannaki, A. (1999). Influence of pH and NaCl on proteolytic and esterolytic activity of intracellular extract of *Leuconostoc mesenteroides* subsp. *mesenteroides* K1G8 Strain. *Milchwissenschaft*, 54, 314-316.

- Wilkinson, M. G., Guinee, T. P., & Fox, P. F. (1994a). Factors which may influence the determination of autolysis of starter bacteria during cheddar cheese ripening. *International Dairy Journal*, 4, 141-160.
- Wilkinson, M. G., Guinee, T. P., O'Callaghan, D. M., & Fox, P. F. (1994b). Autolysis and proteolysis in different strains of starter bacteria during Cheddar cheese ripening. *Journal of Dairy Research*, 61, 249-262.
- Wittenberger, C. L., & Angelo, N. (1970). Purification and Properties of a Fructose-1,6-Diphosphate-Activated Lactate Dehydrogenase from *Streptococcus faecalis*. *Journal of bacteriology*, 101, 717-724.
- Yanachkina, P., McCarthy, C., Guinee, T., & Wilkinson, M. (2016). Effect of varying the salt and fat content in Cheddar cheese on aspects of the performance of a commercial starter culture preparation during ripening. *International Journal of Food Microbiology*, 224, 7-15.

Tables

Table 1

	Protein	Moisture	MNFS ¹	Fat	FDM	Salt	S/M	pH
	(%, w/w)	(%, w/w)	(%, w/w)	(%, w/w)	(%, w/w)	(%, w/w)	(%, w/w)	
<i>L. helveticus</i>	26.76 ^a	36.48 ^a	51.52 ^a	29.69 ^a	46.43 ^a	1.58 ^a	4.74 ^a	5.24 ^b
<i>S. thermophilus</i>	26.31 ^a	36.78 ^a	52.4 ^a	29.50 ^a	46.60 ^a	1.74 ^a	4.66 ^a	5.56 ^a
ST/LH 22% brine	26.46 ^a	36.41 ^a	51.57 ^a	29.52 ^a	46.37 ^a	1.65 ^a	4.81 ^a	5.24 ^b
ST/LH 18% brine	26.32 ^a	37.20 ^a	52.50 ^a	29.40 ^a	46.83 ^a	1.66 ^a	4.60 ^a	5.21 ^b

^(a-b) Means within a column with different superscripts differ ($P < 0.05$). Values presented are the means of four replicates.

¹Abbreviations: MNFS, moisture in the nonfat substance; FDM, fat in dry matter; S/M, salt in moisture; SED, standard error of difference; degrees of freedom = 9

Table 2

		Protein	Moisture	MNFS ¹	Fat	FDM	Salt	S/M	pH
		(%, w/w)	(%, w/w)	(%, w/w)	(%, w/w)	(%, w/w)	(%, w/w)	(%, w/w)	
<i>L. helveticus</i>	Outside	27.33 ^a	34.43 ^c	49.34 ^c	30.23 ^a	45.83 ^c	2.65 ^a	8.12 ^a	5.25 ^b
	Inside	26.19 ^b	38.53 ^a	54.72 ^a	29.14 ^a	47.02 ^a	0.50 ^b	1.14 ^b	5.29 ^b
<i>S. thermophilus</i>	Outside	26.33 ^{a,b}	35.82 ^c	51.54 ^{b,c}	29.39 ^a	45.80 ^c	2.92 ^a	7.92 ^a	5.52 ^a
	Inside	26.31 ^{a,b}	37.74 ^{a,b}	53.26 ^{a,b}	29.61 ^a	47.39 ^a	0.55 ^b	1.33 ^b	5.59 ^a
ST/LH 22% brine	Outside	26.82 ^{a,b}	34.81 ^c	49.89 ^c	29.83 ^a	45.81 ^c	2.71 ^a	8.21 ^a	5.27 ^b
	Inside	26.10 ^b	38.02 ^a	53.25 ^{a,b}	29.19 ^a	46.93 ^{a,b}	0.59 ^b	1.55 ^b	5.21 ^b
ST/LH 18% brine	Outside	26.67 ^{a,b}	35.87 ^{b,c}	50.96 ^c	29.69 ^a	46.18 ^{b,c}	2.72 ^a	7.70 ^a	5.21 ^b
	Inside	25.96 ^b	38.52 ^a	54.03 ^a	29.11 ^a	47.48 ^a	0.46 ^b	0.95 ^b	5.22 ^b

^(a-b) Means within a column with different superscripts differ ($P < 0.05$). Values presented are the means of four replicates.

¹ Abbreviations: MNFS, moisture in the nonfat substance; FDM, fat in dry matter; S/M, salt in moisture; SED, standard error of difference; degrees of freedom = 9

Table 3

		Asp	Thr	Glu	Gly	Ala	Val	Met	Iso	Leu	Tyr	Phe	His	Lys	Arg	Pro
<i>L. helveticus</i>	Out	134 ^{a,b}	144 ^{a,b}	614 ^{b,c}	79 ^{a,b,c}	333 ^b	335 ^{b,c}	93 ^{a,b}	183 ^a	379 ^{b,c}	155 ^{a,b}	264 ^a	218 ^{a,b}	596 ^b	274 ^b	388 ^b
	In	222 ^a	252 ^a	999 ^a	121 ^a	431 ^a	474 ^a	132 ^a	231 ^a	610 ^a	169 ^{a,b}	289 ^a	380 ^a	811 ^a	431 ^a	653 ^a
<i>S. thermophilus</i>	Out	4 ^b	2 ^c	24 ^d	3 ^d	21 ^d	20 ^d	9 ^c	6 ^b	14 ^d	45 ^c	53 ^b	88 ^c	62 ^d	29 ^c	35 ^d
	In	3 ^b	13 ^c	46 ^d	5 ^d	30 ^d	29 ^d	12 ^c	13 ^b	31 ^d	71 ^c	59 ^b	107 ^c	84 ^d	50 ^c	37 ^d
ST/LH 22%	Out	90 ^a	73 ^b	331 ^c	46 ^c	175 ^c	224 ^c	75 ^b	136 ^a	258 ^c	115 ^b	202 ^a	174 ^b	427 ^c	206 ^b	297 ^c
	In	199 ^a	148 ^{a,b}	833 ^{a,b}	88 ^{a,b}	270 ^b	405 ^{a,b}	105 ^{a,b}	230 ^a	588 ^{a,b}	172 ^{a,b}	249 ^a	250 ^a	723 ^a	422 ^a	402 ^b
ST/LH 18%	Out	163 ^a	133 ^{a,b}	425 ^c	48 ^{b,c}	189 ^c	282 ^{b,c}	94 ^{a,b}	171 ^a	407 ^{b,c}	161 ^{a,b}	261 ^a	203 ^{a,b}	530 ^b	279 ^b	259 ^c
	In	110 ^a	260 ^a	880 ^{a,b}	99 ^a	288 ^b	445 ^{a,b}	117 ^{a,b}	252 ^a	673 ^a	223 ^a	301 ^a	234 ^a	817 ^a	443 ^a	501 ^a

Table Legends

Table 1 Chemical composition and pH (7 d of ripening) of experimental cheeses

Table 2 Chemical composition (7 d of ripening) of both sampling areas of experimental cheeses

Table 3 Concentration (mg/kg of cheese) of Free Amino Acids at 26 d of ripening

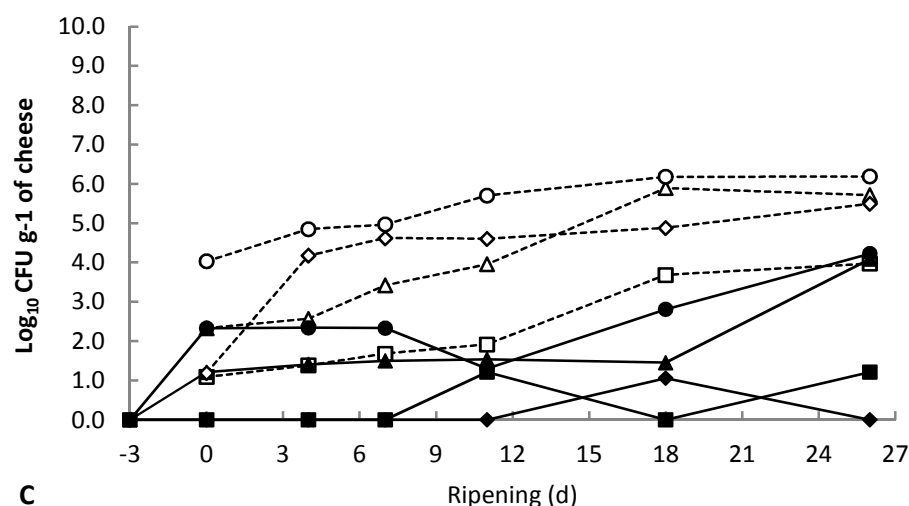
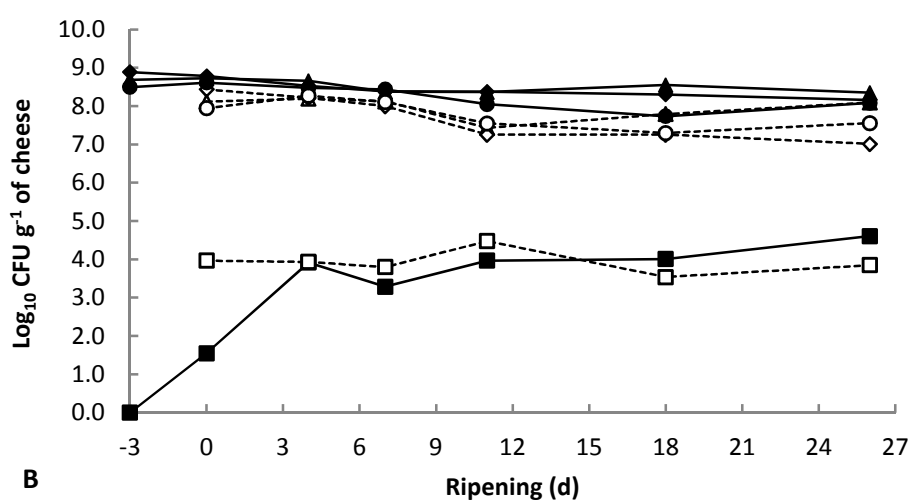
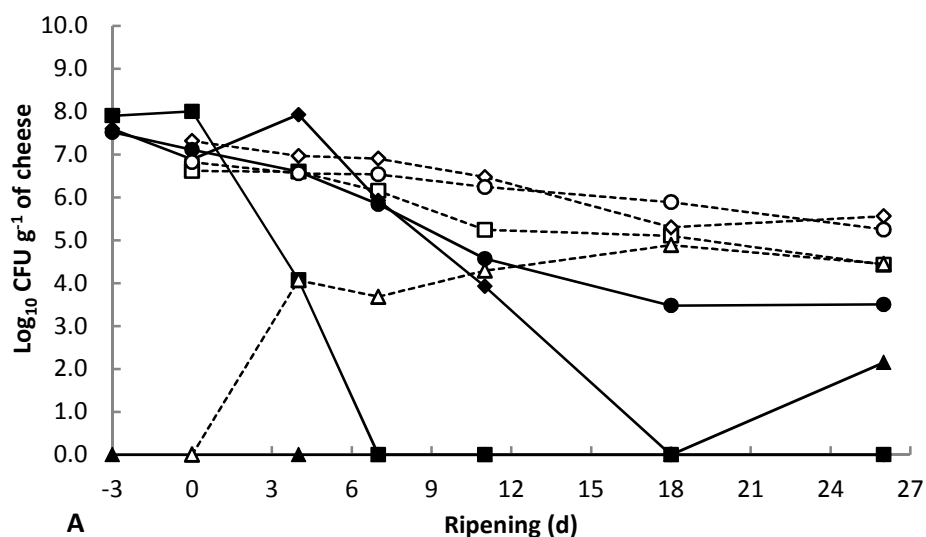


Fig. 1

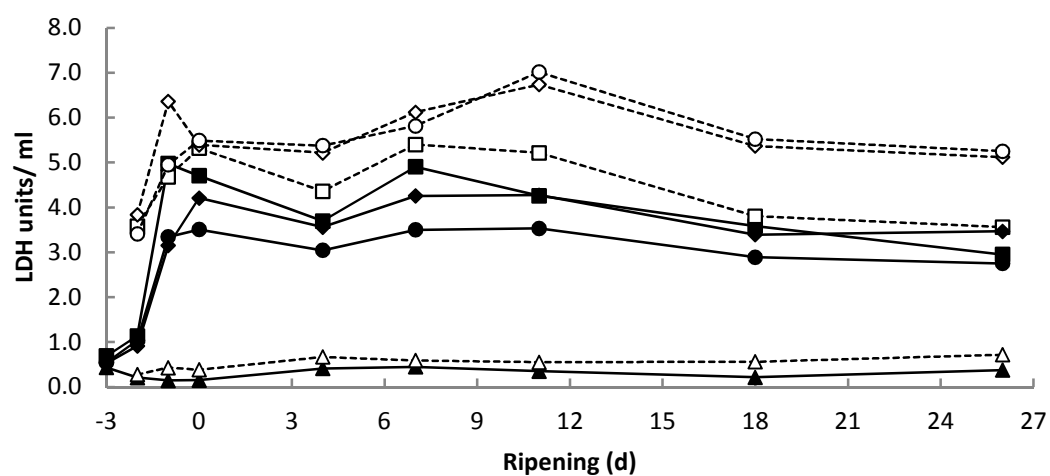
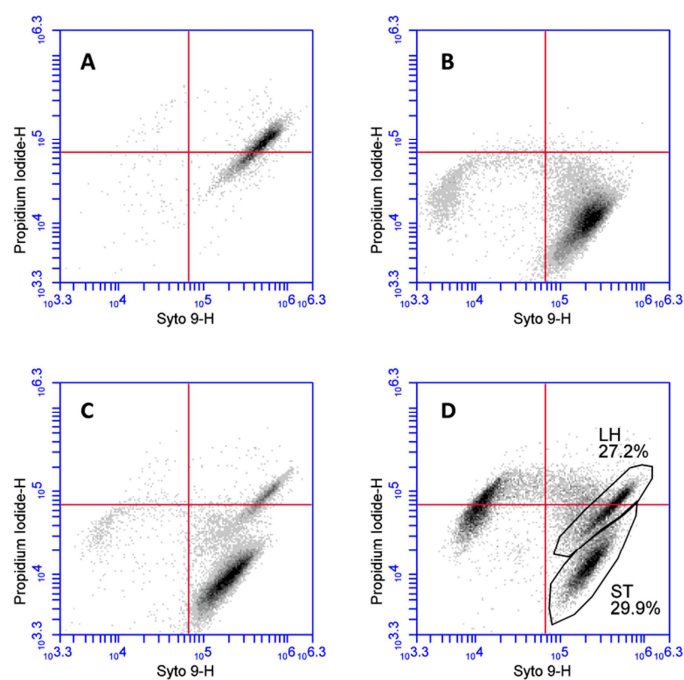
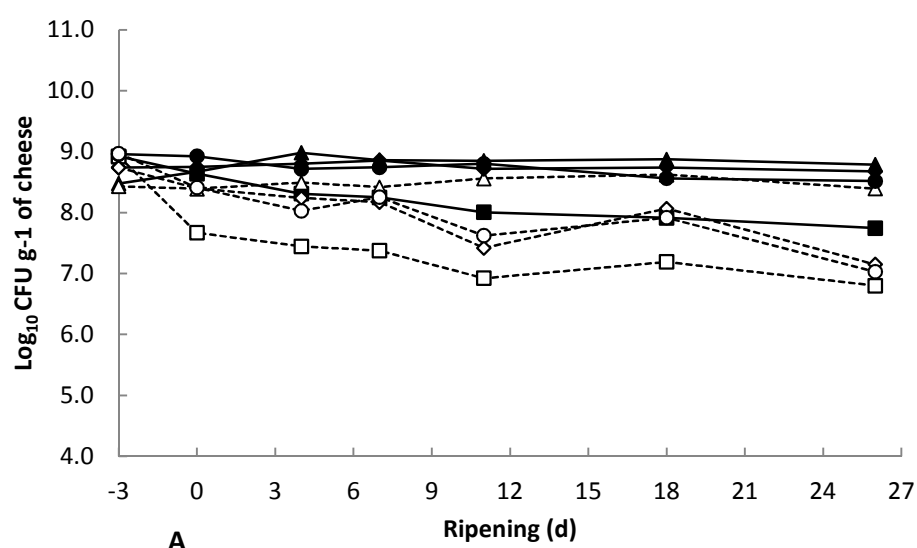
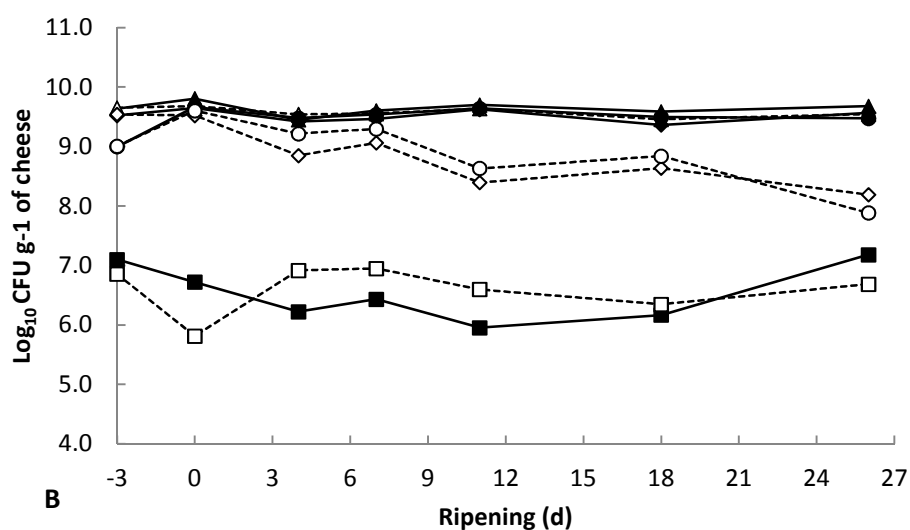


Fig. 2

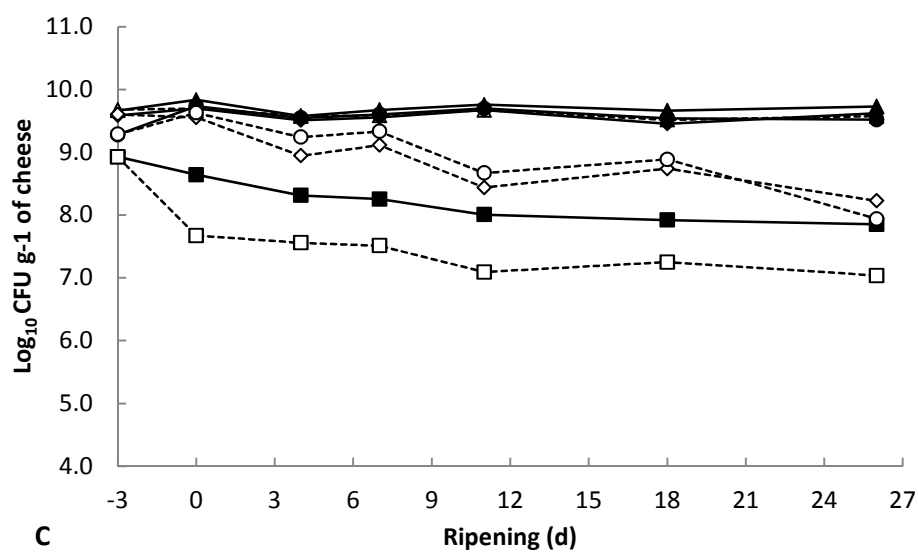
**Fig. 3**



55



56



57

58 Fig. 4

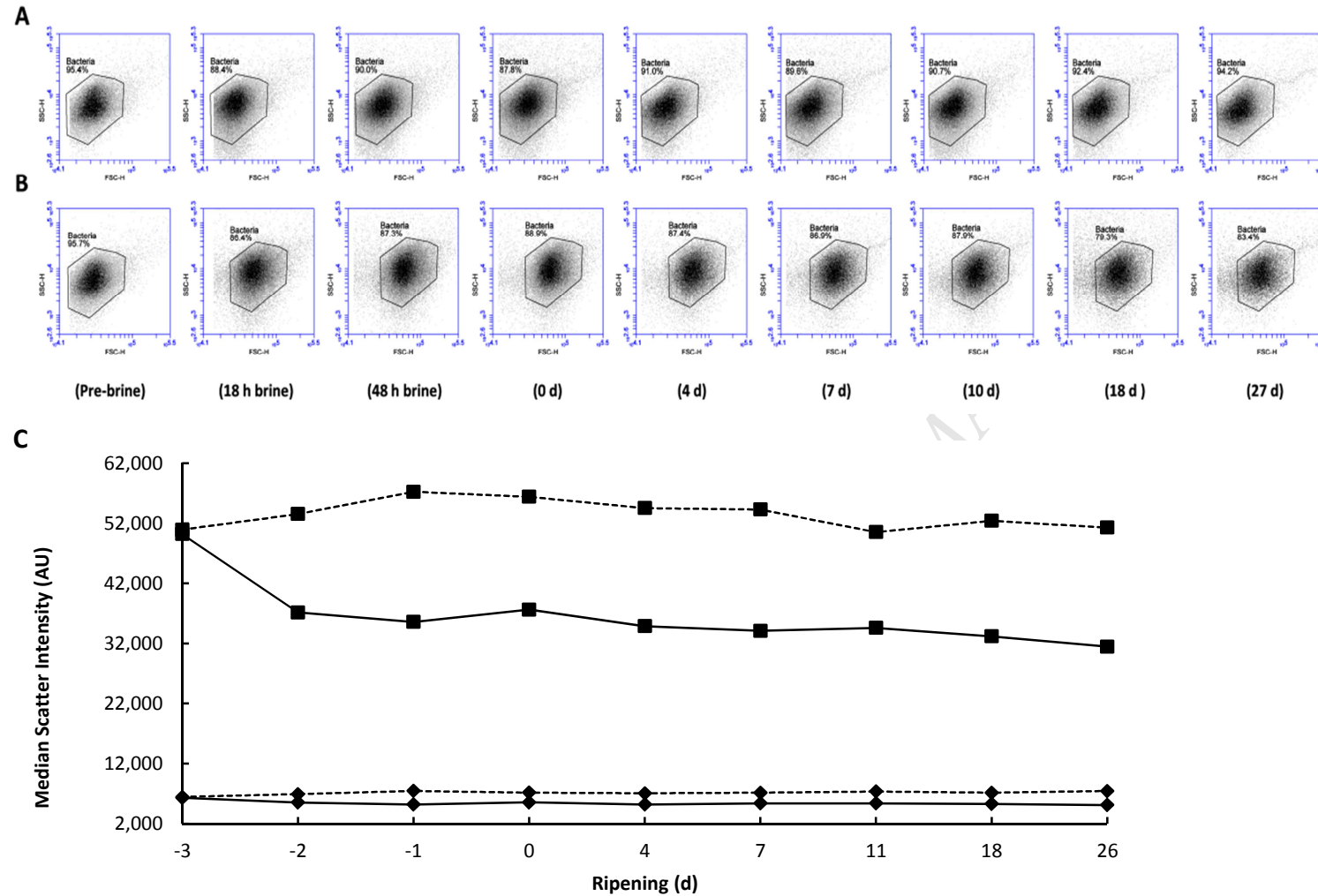


Fig. 5

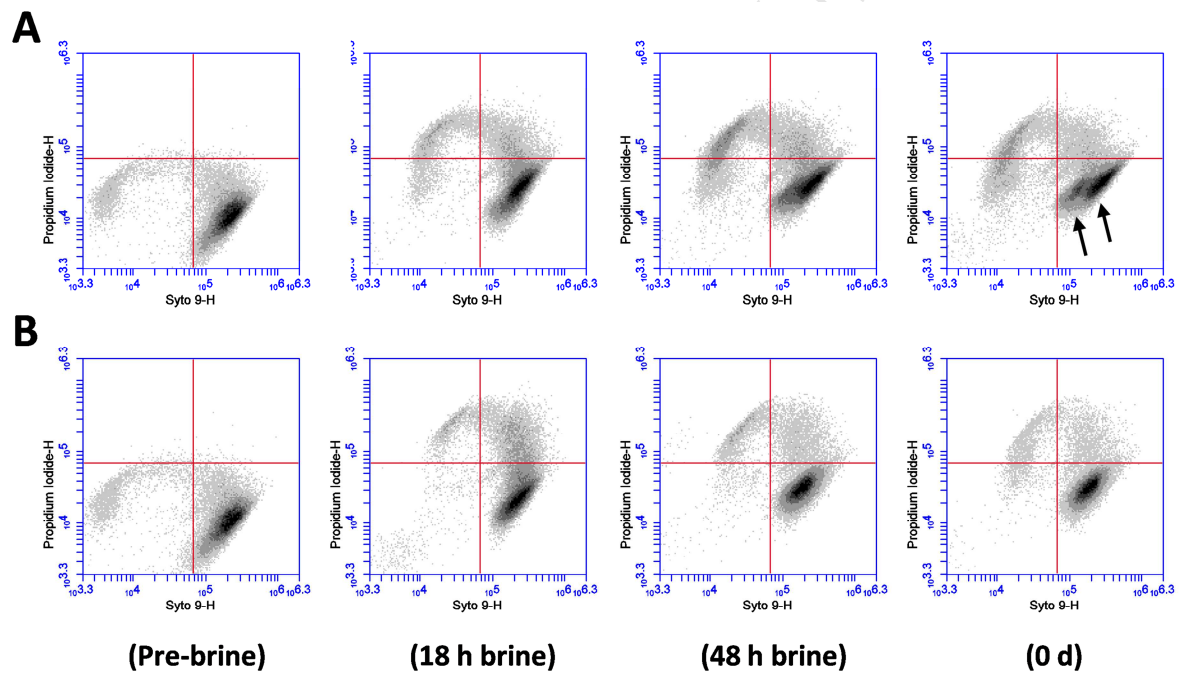
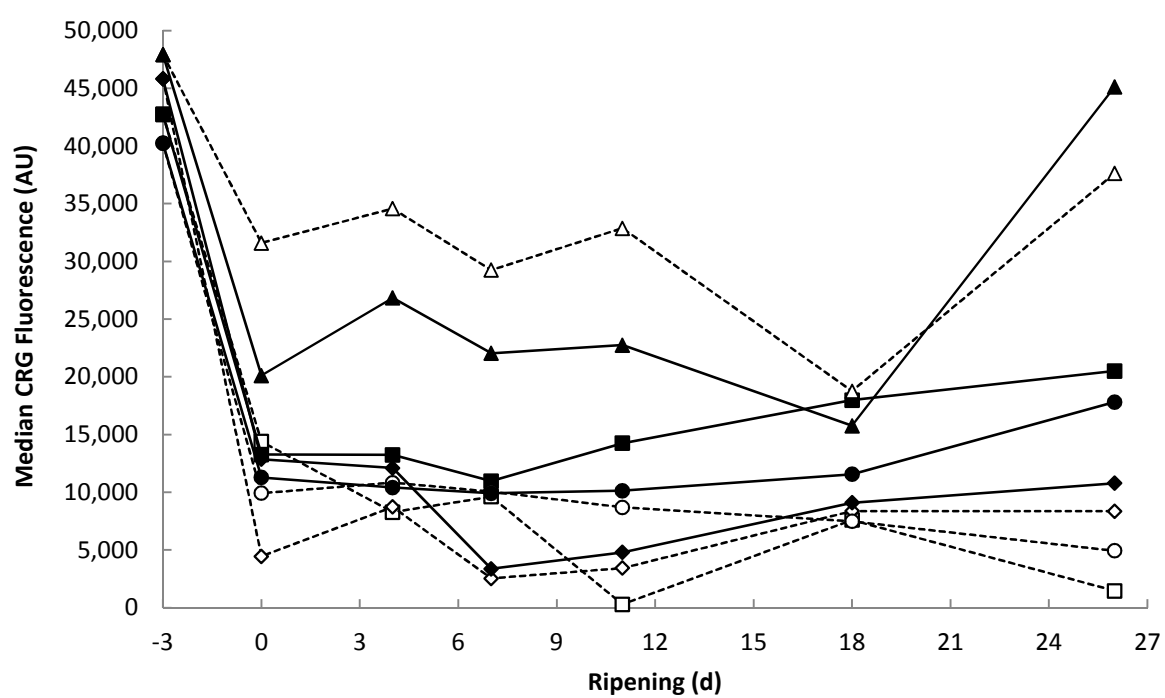


Fig. 6

81



82

83 **Fig. 7.**

84

85

86

87

88

89

90

91

92

93

94

95

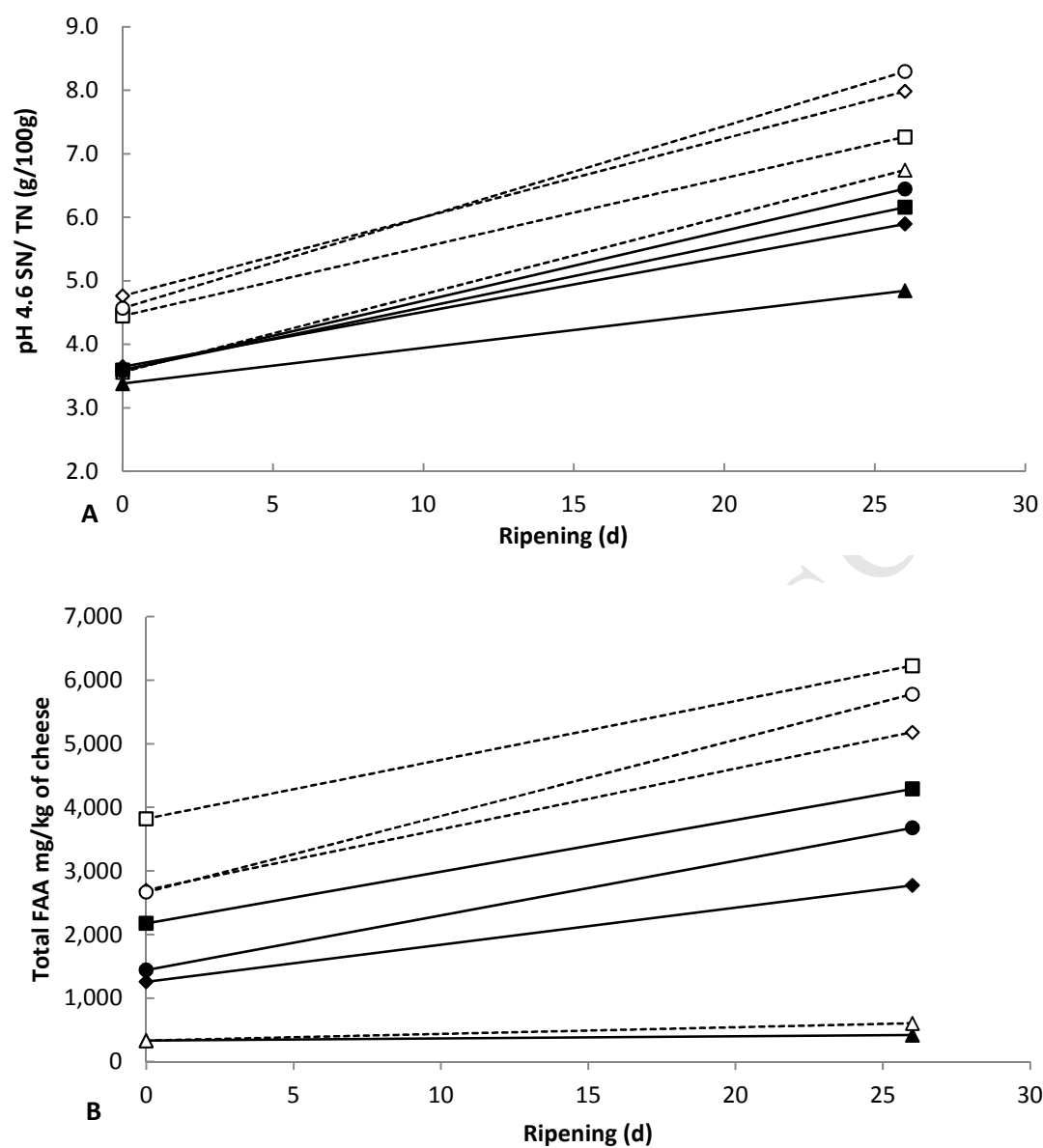


Fig. 8

Figure legends:

Fig. 1. Viable cell counts of (A) *L. helveticus*, (B) *S. thermophilus* and (C) NSLAB in the Outside (—, ■) and Inside (---, □) of experimental cheeses; LH only (■, □), ST only (▲, Δ), ST/LH 22% (◆, ◇), ST/LH 18% (●, ○). Values presented are the means of three replicate trials.

Fig. 2. Levels of lactate dehydrogenase (LDH) release in the Outside (—, ■) and Inside (---, □) of experimental cheeses; LH only (■, □), ST only (▲, Δ), ST/LH 22% (◆, ◇), ST/LH 18% (●, ○). Values presented are the means of three replicate trials.

Fig. 3. Relative positioning of the live populations of *L. helveticus* and *S. thermophilus* starters in Syto9/PI biplots of experimental cheeses: LH-only (A), ST-only (B), ST/LH 22% (C), ST/LH 18% (D). The gating strategy used to enumerate the live populations of *L. helveticus* (LH) and *S. thermophilus* (ST) starters is shown in panel D.

Fig. 4. Live cell counts of (A) *L. helveticus* (gate LH), (B) *S. thermophilus* (gate ST) and (C) combined starters (gates ST + LH), as measured by flow cytometry following Syto9/PI staining, in the Outside (—, ■) and Inside (---, □) layers of experimental cheeses: LH only (■, □), ST only (▲, Δ), ST/LH 22% (◆, ◇), ST/LH 18% (●, ○). Values presented are the means of three replicate trials.

Fig. 5. Flow cytometry analysis of the forward scatter (FSC) and side scatter (SSC) properties of the overall microbiota present in the Inside (A) and Outside (B) cheese layers and of median forward scatter (C) (FSC, ■) and side scatter (SSC, ◆) intensities (AU, arbitrary units) in the Inside (---) and Outside (—) of experimental cheeses. Values presented are the means of three replicate trials.

130

131 **Fig. 6.** Syto9/PI biplots of the overall microflora present in the Outside (A) and Inside (B)
 132 of cheese made using *S. thermophilus* as sole starter. Arrows indicate individual sub-
 133 populations.

134 **Fig. 7.** Flow cytometry temporal analysis of the median CellROX Green (CRG)
 135 fluorescence intensity (AU, arbitrary units) of the overall microflora present in the Outside
 136 (—, ■) and Inside (---, □) of experimental cheeses: LH only (■, □), ST only (▲, Δ),
 137 ST/LH 22% (◆, ◇), ST/LH 18% (●, ○). Values presented are the means of three replicate
 138 trials.

139 **Fig. 8.** Levels of primary proteolysis (A) as measured by % pH4.6 SN/TN and Total free
 140 amino acids (B) in the Outside (—, ■) and Inside (---, □) of experimental cheeses; LH
 141 only (■, □), ST only (▲, Δ), ST/LH 22% (◆, ◇), ST/LH 18% (●, ○). Values presented are
 142 the means of three replicate trials.

Highlights

- High salt results in reduction in size and granularity of microflora in outer layer of brined cheese.
- Cell shrinkage suggestive of internal macromolecule condensation due to hyperosmotic stress.
- Microbiota in the outer cheese layer experienced greater levels of oxidative stress.
- Brining alters physiology of local microbiota.
- Brine salting may result in variations in cheese ripening due to variations in salt concentration.